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Differences in bioactivity between human insulin and insulin analogues approved for therapeutic use- compilation of reports from the past 20 years

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Abstract

In order to provide comprehensive information on the differences in bioactivity between human insulin and insulin analogues, published *in vitro* comparisons of human insulin and the rapid acting analogues insulin lispro (Humalog®), insulin aspart (NovoRapid®), insulin glulisine (Apidra®), and the slow acting analogues insulin glargine (Lantus®), and insulin detemir (Levemir®) were gathered from the past 20 years (except for receptor binding studies). A total of 50 reports were retrieved, with great heterogeneity among study methodology. However, various differences in bioactivity compared to human insulin were obvious (e.g. differences in effects on metabolism, mitogenesis, apoptosis, intracellular signalling, thrombocyte function, protein degradation). Whether or not these differences have clinical bearings (and among which patient populations) remains to be determined.

Introduction

Since the first insulin derivatives were synthetised in the 1970s for scientific purposes [1,2], the therapeutic potential of these compounds for diabetic patients was already under investigation. In those times, there was a quest for metabolically superactive insulins, i.e. "tailor-made" insulin derivatives with enhanced biopotency, like the insulin derivative B10Asp [3], to make the treatment of diabetes mellitus more efficacious [3]. And B10Asp was indeed prepared to be marketed. In 1992 (when the carcinogenicity of B10Asp [4] was disclosed by haphazard) it was realized that manipulations of the insulin molecule could introduce the risk of artificial bioactivities into the treatment of diabetic patients [4-6]. Probably because of such concerns, insulin manufacturers subsequently favoured the design of derivatives (euphemistically called insulin analogues) with particular absorption properties (i.e. faster or more prolonged absorption from the subcutaneous tissue after injection [7,8]). Since 1996, five of such insulin analogues have been approved for human use (the rapid acting analogues aspart, glulisine and lispro, and the slow-acting analogues glargine and detemir). Their biological potencies still remain to be fully elucidated [4].

In order to expose the known differences in all fields of bioactivity between these insulin analogues and human insulin, we registered all *in vitro* studies - except for receptor binding studies - published from 1990 to 2010 and displayed their findings schematically in the following report.

Materials and methods

An electronic search was conducted in the PubMed database using the following key words: *in vitro*, proliferation, mitogenic, mitogenicity, metabolic, apoptosis, potency, glucose transport, lipogenesis, intracellular signalling, in conjunction with the currently marketed insulin analogues searched for by their international non-proprietary names: insulin aspart [B28Asp human insulin] (Novo-Nordisk, Bagsvaerd, Denmark), insulin detemir [B29Lys (epsilon-tetradecanoyl), desB30 human insulin] (Novo-Nordisk, Bagsvaerd, Denmark), insulin glargine [A21Gly, B31Arg,B32Arg human insulin] (Sanofi-Aventis, Paris, France), insulin glulisine [B3Lys,B29Glu human insulin] (Sanofi-Aventis, Paris, France) and insulin lispro [B28Lys, B29Pro human insulin] (Eli Lilly, Indianapolis, IN, USA). Their respective brand names Humalog®, NovoRapid®, Lantus®, Apidra®, Levemir® and their company abbreviations HOE 901(= Lantus®), NN 304(= Levemir®), HMR 1964 (= Apidra®), B28Asp (= NovoRapid®) and LY275585(= Humalog®) were also searched for. A hand

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search was performed on reference lists, published symposium reports, and abstract volumes of scientific meetings. The time span covers the years 1990 to 2010. Receptor binding studies were not addressed in this search. The publications are reported in a schematic fashion, in alphabetical order of the analogues' international non-proprietary names.

Results

Altogether, 50 publications were retrieved reporting *in vitro* data on comparisons between human (native) insulin and one of the insulin analogues indicated above [5,9-57]. There were 45 full papers [5,9,21,23-27,31-38,40-57] and 5 abstracts [22,28-30,39]. The publications were screened for differences in seven categories: metabolic activity, mitogenic activity, anti-apoptotic activity, intracellular signalling, effects on thrombocytes, effects on protein degradation, and intracellular internalization. The studies were performed or sponsored by pharmaceutical companies (n = 27) or independent institutions [10,12,16-18,27,31-34,37,39,41-43,55-57]; in 3 studies, possible sponsoring could not be identified.

Differences in metabolic activity

There were 14 publications reporting *in vitro* studies on the metabolic activity of insulin analogues (assessed in primary mouse adipocytes, primary rat cardiomyocytes and adipocytes, human muscle cells, human dermal microvascular endothelial cells, 3T3-L1 adipocytes, and L6 myocytes) in comparison to synthetic human insulin.

Aspart

This issue was addressed in the Scientific Discussion of insulin aspart, published by the European Medicines Agency (EMEA) (page 4: "in mouse free fat cells, the stimulation of lipogenesis did not differ between insulin aspart and human insulin, lending further support to similar molar potency" [58]). In primary rat adipocytes, the effects on glucose transport and lipogenesis were similar to human insulin [21], and in primary mouse adipocytes the effect on lipogenesis was similar to human insulin [26,53].

Detemir

This issue was addressed in the Scientific Discussion of insulin detemir, published by the EMEA (pages 1-6: "insulin detemir was consistently less potent than human insulin in all *in vitro* assays. Depending on the assay, the potency was 2- to 10-fold lower" [59]). In primary mouse adipocytes [26] and primary rat adipocytes [48], in 3T3-L1 adipocytes [12,54] and in L6 myocytes [54] the metabolic potency was only 1-20% of that of human insulin by equimolar comparison. (Therefore, the manufacturer increased the concentration of the active substance in the vial four-fold compared to synthetic human (or porcine

natural) insulin [48]: 1 unit of human insulin is 6 nmol insulin, while 1 unit of insulin detemir is 24 nmol [59]).

Glargine

This issue was addressed in the Scientific Discussion of insulin glargine, published by the EMEA (page 5: "nearly all *in vitro* metabolic studies have shown a relative *in vitro* potency for insulin glargine of about 50% and similar maximal responsiveness as compared to human insulin....there is evidence that higher plasma levels *in vivo* compensate for the 50% *in vitro* potency found for insulin glargine as compared to human insulin"[60]). In primary mouse adipocytes [26], primary rat cardiomyocytes [9,46] and in 3T3-L1 adipocytes [19,25] metabolic potency was only about 50% of that of human insulin by equimolar comparison. Another study in 3T3L1 adipocytes showed a similar metabolic potency to human insulin [55]. In non-starved muscle cells, the effect on glucose uptake was similar to human insulin [14]. In human dermal microvascular endothelial cells, there was no effect, similar to human insulin [13].

Glulisine

This issue was addressed in the Scientific Discussion of insulin glulisine, published by the EMEA (page 8: "Stimulation of glucose transport was equal for both insulin glulisine and human insulin...lipogenic activity and glucose transport in isolated rat adipocytes was slightly lower than human insulin, but reached the same maximum obtainable effects as human insulin at higher concentrations"[61]). In rat cardiomyocytes, the effect on glucose transport [35], and in non-starved muscle cells, the effect on glucose uptake [15] was similar to human insulin.

Lispro

This issue was addressed in the Scientific Discussion of insulin lispro, published by the EMEA (page 2: "Insulin lispro is biologically equivalent to insulin in several *in vitro* tests, including ...glucose transport in adipocytes" [62]). In primary mouse adipocytes [26] the effect on lipogenesis was comparable to that of human insulin. A study in L6 myotubes showed a similar metabolic potency compared to human insulin [47].

Differences in mitogenic activity

As standard 2-year carcinogenicity *in vivo* studies in animals (see EMEA: Note for Guidance on Carcinogenic Potential; CPMP/SWP/2877/00) have not been performed with any of the commercially available insulin analogues (except for insulin glargine [58-62]), the issue of mitogenicity has been addressed by quite a few *in vitro* investigations. There were 34 publications reporting *in vitro* data on the mitogenic activity of insulin analogues with various non-malignant cell types: primary muscle cells, human smooth muscle cells, Chinese hamster ovary cells (CHO-K1), human dermal microvascular

endothelial cells, human mammary epithelial cells, rat-H9c2 myoblasts, H9 cardiac myoblasts, rat-1 fibroblasts, rat H4-II-E hepatoma cells, K6 myoblasts, L6 myoblasts, HepG2 cells, C2C12 myoblasts, mammary gland-derived MCF-10 cells, human coronary artery endothelial cells, human coronary artery smooth muscle cells, primary human smooth muscle cells, vascular smooth muscle cells, primary mouse mammary gland epithelial cells, human lung fibroblast HEL-299 cells, and rat thyrocytes FRTL-6. Various malignant cell types were also studied: human osteosarcoma cells (Saos/B10 cell, Saos-2 cells), human mammary carcinoma cells (MCF-7 cells, T47 D cells, MDA-MB 231 cells, SKBR-3 cells), human colonic carcinoma cells HCT-116, pancreatic carcinoma cells Colo-357, prostate carcinoma cells PC-3, thyroid cancer cells FTC-133, and human bladder cancer cells T 24. The experiments were performed in comparison with synthetic human insulin.

Aspart

This issue was addressed in the Scientific Discussion of insulin aspart, published by the EMEA (page 4: "The results in CHO K1 cells were essentially similar to those of human insulin whereas the mitogenic activity of insulin aspart in MCF-7 cells indicated differences to human insulin. Subsequent analysis indicated lower activity than initially calculated but the analysis also showed that the results in MCF-7 cells were not sufficiently robust for proper assessment. Newly performed studies using human osteosarcoma B10 cells revealed essentially similar response of insulin aspart and human insulin" [58]).

In four studies using non-malignant cells (muscle cells, CHO-K1 cells, MCF-10 A cells) insulin aspart showed a similar mitogenic potency, compared to human insulin [5,21,32,41,42]. In malignant MCF-7 cells and T 47 D cells, insulin aspart showed similar mitogenic potency as human insulin [32]. In malignant Saos/B10 cells, insulin aspart showed lower mitogenic potency [26]. In one study using the malignant MCF-7 cells, insulin aspart showed a non-significantly higher mitogenic potency compared to human insulin [41,42].

Detemir

This issue was addressed in the Scientific Discussion of insulin detemir, published by the EMEA (page 8: "Relative to human insulin, the albumin-corrected mitogenic potency was 9% in CHO-K1 cells, 15% in a human mammary cancer cell line (MCF-7 cells), 11% in a human osteosarcoma cell line, and 25% in L6-IhR cells. Based on these data, the mitogenic potency of insulin detemir *in vitro* seems to be reduced relative to human insulin to approximately the same extent as its binding affinity for the insulin and IGF-1 receptors" [59]). Ten years later, however, the company emphasized that "the potency estimates are different between the *in vivo* and *in vitro*

conditions" [48]. There were nine studies which had equimolar comparisons to human insulin, and one that was based on equipotent comparison [33].

In five studies using non-malignant cells (vascular smooth muscle cells [54], MCF-10A cells [32,41,42], HEL-299 cells [55]), insulin detemir showed similar mitogenic potency compared to human insulin. In one study using non-malignant human muscular endothelial cells [22] and L6-hlR cells [22], the mitogenic potency of insulin detemir was non-significantly lower compared to human insulin. Using the malignant MCF-7 cells, insulin detemir showed similar mitogenic potency in two studies [32,41,42], and higher mitogenic potency in one study [56]. Studies using the malignant Saos/B10 cells [26], and T24 cells [30] showed less mitogenic potency of insulin detemir, and a study using HCT-116 cells [56] and PC-3 cells [56] showed higher mitogenic potency of insulin detemir compared to human insulin. In the one study using MCF-7 cells with equipotent comparison, insulin detemir showed similar mitogenic potency compared to human insulin [33].

Glargine

This issue was addressed in the Scientific Discussion of insulin glargine, published by the EMEA (page 7: "... insulin glargine might have a mitogenic potential through binding to the IGF-1 receptor. This point of concern was addressed in an oral explanation at the Committee for Proprietary Medicinal Products (CPMP) where the company highlighted that insulin glargine had a lower mitogenic activity than the comparator B10-Asp insulin or IGF-1 in 3 out of 4 cell assays"[60]). There were 26 studies on this topic, all with equimolar comparisons to human insulin.

In 12 studies using non-malignant cells (H9 rat cardiomyoblasts [9,28], rat-1 fibroblasts [11], primary human muscle cells [14], primary human smooth muscle cells [16], human coronary artery smooth muscle cells [49], human coronary artery endothelial cells [49], vascular smooth muscle cells [54], MCF-10 A cells [32,41,42], rat thyrocytes FRTL-5 [34] and HepG2 cells [19]), insulin glargine showed similar mitogenic potency compared to human insulin. In 5 studies using non-malignant cells (human dermal microvascular endothelial cells [13], human mammary epithelial cells [22,25], individual primary human smooth muscle cells [16], HEL-299 cells [55]), insulin glargine showed higher mitogenic potency compared to human insulin. In one study using the non-malignant L6-hlR cells [22], insulin glargine showed lower mitogenic potency compared to human insulin.

In 6 studies using malignant cells (MCF-7 cells [50], Colo-357 cells [17], T47 D cells [32], MDA-MB-231 cells [29], T24 cells [31], thyroid cancer FTC-133 cells [34], insulin glargine displayed similar mitogenic potency as compared to human insulin. In 12 studies using malignant

cells (Saos/B10 cells [21,26,39,46], MCF-7 cells [27,32,33,41,42,56], SKBR-3 cells [27], HCT-11 cells [56] and PC-3 cells [56], insulin glargine showed increased mitogenic potency compared to human insulin. In one study using Saos/B10 cells, the insulin glargine metabolite IM showed increased mitogenic potency [46], while the glargine metabolites M1 and M2 showed similar mitogenic potency compared to human insulin [46].

Glulisine
This issue was addressed in the Scientific Discussion of insulin glulisine, published by the EMEA (page 9: "(thymidine incorporation) was equal for insulin glulisine and human insulin.." [61]).

Six studies using non-malignant cells (K6 myoblasts [35], C2C12 myoblasts [23], primary human muscle cells [15], rat-1 fibroblasts [51], MCF-10 A cells [32,43] and mammary gland cells [43]) showed equal mitogenic potency of insulin glulisine and human insulin, whereas one study using MCF-10 cells showed less mitogenic potency of insulin glulisine [24].

Two studies in malignant MCF-7 cells [32,43] showed equal mitogenic potency of insulin glulisine and human insulin.

Lispro
This issue was addressed in the Scientific Discussion of insulin lispro, published by the EMEA (page 2: "In cell growth assays using human smooth muscle cells and human mammary epithelial cells and using... thymidine incorporation or increases in cell number as index of cell growth, insulin lispro was shown to be equipotent to human insulin"[62]).

In five studies using non-malignant cells (human mammary epithelial cells [45], rat H4-II-E hepatoma cells [20], and MCF-10A cells [32,41,42], insulin lispro displayed similar mitogenic potency compared to human insulin. In one study using human smooth muscle cells [44], insulin lispro displayed higher mitogenic potency, and in another study using human smooth muscle cells, insulin lispro displayed lower mitogenic potency compared to human insulin [44].

In three of the studies using malignant cells (MCF-7 [32,41,42] and T 24 D [32]), insulin lispro displayed equal mitogenic potency compared to human insulin; in one study using Saos/B10 cells [26], insulin lispro showed lower, and in one study using HCT-116 cells [56], insulin lispro showed higher mitogenic potency compared to human insulin.

Differences in anti-apoptotic activity
This issue was not specifically addressed in the Scientific Discussions of any of the five analogues published by the EMEA [58-62]). However, there were six publications [17,36,49,52,56,57] reporting *in vitro* data on the

inhibition of apoptosis by insulin analogues versus human insulin.

Aspart
There was only one study of insulin aspart using malignant INS-1 cells [36]; the inhibition of apoptosis was similar compared to human insulin.

Detemir
There were two studies of insulin detemir, conducted with equimolar comparisons to human insulin in malignant HCT-116 cells [56,57]; in both studies, insulin detemir inhibited apoptosis more than did human insulin.

Glargine
There was one study using non-malignant cells (human coronary endothelial cells [49] and human coronary artery smooth muscle cells [49]) showing no effect on apoptosis by either insulin glargine or human insulin. In one study conducted with equimolar concentrations of insulin glargine and human insulin in malignant Colo-357 cells [17], inhibition of apoptosis was similar between both compounds. In malignant HCT-116 cells [56,57], and malignant INS-1 cells [52], insulin glargine inhibited apoptosis stronger compared to human insulin.

Glulisine
There were two studies of insulin glulisine, conducted in malignant INS-1 cells [36,52], showing stronger inhibition of apoptosis by insulin glulisine compared to human insulin.

Lispro
There were two studies of insulin lispro, conducted in malignant INS-1 cells [36,52], one showing similar inhibition [52], and one showing stronger [36] inhibition of apoptosis by insulin lispro compared to human insulin.

Differences in intracellular signaling
The issue was not specifically addressed in the Scientific Discussions of any of the five analogues published by the EMEA [58-62]. However there were 12 publications [14,23,31,35,40-43,47,54,56,57] reporting *in vitro* data on intracellular signalling by insulin analogues versus human insulin concerning the Akt-GSK-3 pathway (involved in metabolic activity), and the Erk-MAPK pathway (involved in proliferative activity).

Aspart
There were two studies of insulin aspart, conducted in non-malignant MCF-10A cells and malignant MCF-7 cells [41,42], and in IGF-1 receptor-deprived mouse fibroblasts transfected with either the human insulin receptor A or B isoforms [40], all of which showed equal Akt-activation and GSK-3 inactivation of insulin aspart compared to human insulin. Erk 1/Erk 2 phosphorylation by insulin aspart was similar to human insulin in MCF-10A cells [41,42], but was increased in fibroblasts

expressing insulin receptor A isoform [40], and decreased in fibroblasts expressing insulin receptor B isoform [40].

Detemir

There were five studies of insulin detemir conducted with equimolar comparisons to human insulin [41,42,54,56,57], and one with equipotent comparison [40].

A similar Akt-activation by insulin detemir and human insulin was found in non-malignant MCF-10A cells [41,42] and in non-malignant fibroblasts expressing insulin receptor A isoform [40]. A weaker Akt-activation by insulin detemir compared to human insulin was found in non-malignant cells (3T3-L1 adipocytes [54], L6 myocytes [54], primary hepatocytes [54], primary vascular smooth muscle cells [54]), and in non-malignant fibroblasts expressing insulin receptor B isoform [40].

In non-malignant cells (3T3-L1 adipocytes [54], L6 myocytes [54], primary hepatocytes [54], primary vascular smooth muscle cells [54]) GSK-3 inactivation by insulin detemir was weaker than that by human insulin. In non-malignant MCF-10A cells, GSK-3 inactivation was similar by insulin detemir and human insulin [41,42]. Erk 1/Erk 2 phosphorylation by insulin detemir was equal to that by human insulin in two studies using non-malignant cells (MCF-10A cells [41,42] and fibroblasts expressing insulin receptor B isoform [40]. Erk 1/Erk 2 phosphorylation was increased by insulin detemir in fibroblasts expressing insulin receptor A isoform [40].

In non-malignant cells (3T3-L1 adipocytes [54], L6 myocytes [54], primary hepatocytes [54], primary vascular smooth muscle cells [54]), MAP-kinase activation by insulin detemir was weaker than that by human insulin. In malignant cells (MCF-7 [41,42], HCT-116 [56,57]), Akt-activation by insulin detemir was weaker than that by human insulin. In MCF-7 cells, GSK-3 inactivation was weaker by insulin detemir as compared to human insulin [41,42]. Erk 1/Erk 2 phosphorylation was decreased by detemir in MCF-7 cells [41,42] and HCT-116 cells [56,57]. In MCF-7 cells, MAP-kinase activation by detemir was similar to that by human insulin [41,42].

Glargine

There were eight studies of insulin glargine, all of which with equimolar comparisons to human insulin [14,31,40-42,54,56,57]. Akt-activation by insulin glargine was similar to that by human insulin in non-malignant primary human muscle cells [14], 3T3-L1 adipocytes [54], L6 myocytes [54], primary hepatocytes [54], primary vascular smooth muscle cells [54], and in fibroblasts expressing insulin receptor A isoform [40]. In non-malignant MCF-10A cells, Akt-activation by insulin glargine was stronger [41,42], and in fibroblasts expressing insulin receptor B isoform it was weaker than that by human insulin [40]. GSK-3 inactivation by insulin glargine was similar to that by human insulin in non-malignant 3T3-L1 adipocytes

[54], L6 myocytes [54], primary hepatocytes [54], primary vascular smooth muscle cells [54], and MCF-10A cells [41,42]. Erk 1/Erk 2 phosphorylation by insulin glargine was equal to that by human insulin in fibroblasts expressing insulin receptor B isoform [40], it was stronger in fibroblasts expressing insulin receptor A isoform [40], and weaker in MCF-10A cells [41,42]. MAP-kinase activation by glargine was equal to that by human insulin in primary human muscle cells [14] and in primary rat vascular smooth muscle cells [54].

Glulisine

Akt-activation by insulin glulisine was similar to that by human insulin in non-malignant rat cardiomyocytes [35], and in fibroblasts expressing insulin receptor A isoform [40]. Akt-activation by insulin glulisine was weaker than that by human insulin in MCF-10A cells [43], in mouse muscle tissue [23], in mouse liver tissue [23], and in fibroblasts expressing insulin receptor B isoform [40]. GSK-3 inactivation by insulin glulisine was similar to that by human insulin in rat cardiomyocytes [35], and weaker in MCF-10A cells [43]. Erk 1/Erk 2 phosphorylation by insulin glulisine was similar to that by human insulin in MCF-10 cells [43], and weaker in K6 myoblasts [35], in fibroblasts expressing insulin receptor A isoform [40], and in fibroblasts expressing insulin receptor B isoform [40]. MAP-kinase activation by glulisine was similar to that by human insulin in mouse muscle tissue [23], and mouse liver tissue [23].

Lispro

Akt-activation by insulin lispro was similar to that by human insulin in non-malignant MCF-10 A cells [41,42], and in fibroblasts expressing insulin receptor A isoform [40]. Akt-activation by insulin lispro was weaker than that by human insulin in fibroblasts expressing insulin receptor B isoform [40]. GSK-3 inactivation by lispro was similar to that by human insulin in MCF-10A cells [41,42]. Erk 1/Erk 2 phosphorylation by insulin lispro was similar to that by human insulin in L6 myocytes [47], and in MCF-10A cells [41,42]; it was weaker in fibroblasts expressing insulin receptor B isoform [40], and stronger in fibroblasts expressing insulin receptor A isoform [40].

The studies mentioned above are listed in Table 1.

Differences in effects on thrombocytes

The issue was not specifically addressed in the Scientific Discussions of any of the five analogues published by the EMEA [58-62]. However, there were two publications [37,38] that compared the effects of insulin analogues and human insulin on thrombocyte function.

Aspart

Inhibition of platelet aggregation was stronger than that by human insulin; both compounds affected platelet aggregation via PI-3 K activation [37].

Table 1 Bioactivities of insulin analogues versus human insulin

		Aspart	Detemir	Glargine	Glulisine	Lispro
metabolic activity	lower		12,26,48,54	9,19,25,26		
	equal	21,26,53		13,14,55	15,35	26,5
	higher					
mitogenic activity	lower	26	22,26,30	22		26,44
	equal	5,21,32,41,42	32,33,41,42,54,55	9,11,14,16,17,19,28,29,31, 32,34,41,42,49,50,54	15,23,32,35,43,51	20,32,41,42,45
	higher	41,42	56	13,16,21,22,25-27,32,33,39, 41,42,46,55,56		44,56
apoptosis inhibition	lower					
	equal	36		17,49		52
	higher		56,57	52,56,57	36,52	36
Akt-activation	lower		40-42,54,56,57	40	23,40,43	40
	equal	40-42	40-42	14,40,54	35,40	40-42
	higher			41,42		
GSK-3 inactivation	lower		41,42,54	41,42,54	43	41,42
	equal	40-42	41,42		35	
	higher					
Erk-phosphorylation	lower	40	41,42,56,53	41,42	23,40,43	40
	equal	41,42	40-42	40	35,40	41,42,47
	higher	40	40	40		40
MAP-kinase activation	lower		54			
	equal		41,42	14,54	23	
	higher					

Compilation of reports (numbers refer to the publications cited in the reference list) on bioactivities of the insulin analogues aspart, detemir, glargine, glulisine, and lispro versus human insulin.

Lispro
Inhibition of platelet aggregation was stronger than that by human insulin; both compounds affect platelet aggregation via PI-3 K activation [38].

Differences in effects on protein degradation
This issue was not specifically addressed in the Scientific Discussion of any of the five analogues published by the EMEA [58-62]. However, there were two publications [18,19] on this topic.
Glargine
Inhibition of protein degradation in HepG2 cells was less than that by human insulin [19].
Lispro
Inhibition of protein degradation in H4-II-E hepatoma cells was much stronger than that by human insulin, and inhibition of protein degradation in Hep2G cells and in L6 cells was similar to that by human insulin [18].

Differences in intracellular internalization and degradation
The issue was not specifically addressed in the Scientific Discussion of any of the five analogues published by the EMEA [58-62]. However, there were four publications [10,19,20,35] on this topic.
Glargine
Internalization into HepG2 cells was similar to that of human insulin, whereas degradation was much less compared to human insulin [19].
Glulisine
Internalization into and degradation by K6 rat myoblasts was less compared to human insulin [35].
Lispro
Internalization into rat hepatocytes was similar to that of human insulin [20]; degradation by insulin degrading enzyme (IDE) was similar to that of human insulin [10,20].

Miscellaneous
Effect on gene expression
This issue was not specifically addressed in the Scientific Discussion of any of the five analogues published by the EMEA [58-62]. However, there were three publications [12,41,43] on this topic.
Detemir
In 3T3-L1 preadipocytes, the effect of insulin detemir on the gene expression of leptin and PPAR-Gamma-2

was considerably reduced compared to human insulin [12].

Glargine
In MCF-7 cells, insulin glargine induced a higher expression of the Cyclin D1 gene, compared to human insulin [41].

Glulisine
In HepG2 cells, neither insulin glulisine nor human insulin induced hexokinase-4 expression [43]. In HepG2 cells, hexokinase-2 expression was induced to the same extent by both 150 nmol human insulin and insulin glulisine; no effect of either compound at lower concentrations was observed in HepG2 cells or MCF-7 cells [43].

Addendum: differences in bioactivity between native porcine or bovine insulin and synthetic human insulin
For comparison, the bioactivities of synthetic human insulin versus native animal insulin (bovine and porcine) are reported, as retrieved from six publications [32,41-43,63,64].

Metabolic potency
In human and rat adipocytes, porcine insulin showed slightly higher metabolic potency (in terms of lipogenesis in rat epididymal and human subcutaneous fat cells), as compared to synthetic (recombinant DNA) human insulin (Humulin®, Eli Lilly, Indianapolis, IN, USA) [64].

Mitogenicity
In MCF-7 cells, bovine insulin showed slightly less mitogenic potency compared to synthetic human insulin (Actrapid®, NovoNordisk, Bagsvaerd, Denmark), whereas in MCF-10A and T47 D cells, bovine insulin showed a similar mitogenic potency to human insulin [32,41-43]. In bovine dermal fibroblasts GM06034 and GM06035, bovine insulin and synthetic human insulin (Humulin®) displayed a similar mitogenic potency [63].

Intracellular signalling
In MCF-7 and MCF-10A cells, bovine insulin showed a slightly weaker Erk 1/2 phosphorylation compared to synthetic human insulin (Actrapid®), whereas the effect on Akt-phosphorylation and GSK 3 alpha/beta-phosphorylation was similar to that of synthetic human insulin [41-43].

Gene expression
In HepG2 cells, neither bovine nor human insulin induced hexokinase 4 expression [43].

Discussion
Our schematic presentation shows that in the 1990s most *in vitro* studies were industry-sponsored ones (except for study [5]), and were performed using non-malignant cells, whereas in later years, the majority of studies were performed using malignant cells, and many

of them were carried out by industry-independent investigators. This change in direction may have been brought about by the *Points to Consider* Document of the EMEA [65] of 2001, which particularly requested studies using malignant cell lines, or malignant tissues, or animal models of malignancies to prove the safety of insulin analogues in terms of tumour growth promotion. The rationale for this request was that non-malignant and malignant tissues may respond differently to stimulation by insulin or insulin analogues, due to differences in the expression (or the function) of insulin receptors and IGF-1 receptors between non-malignant and malignant tissues.

Moreover, we found that the metabolic bioactivity of insulin analogues was studied exclusively on benign primary fat cells, or fat cell lines. According to these investigations, the analogue insulins aspart, glulisine and lispro were shown to have equal metabolic potencies to human insulin, whereas the analogues glargine and detemir considerably reduced metabolic activity. The growth-promoting bioactivity, e.g. effects on mitogenicity and apoptosis, showed equal potencies of the insulin analogues and human insulin in most circumstances-when studied in benign cells. Different effects, particularly on mitogenicity and apoptosis, only became apparent when the insulin analogues and human insulin were studied in malignant cells. Intracellular signalling was examined for all of the insulin analogues, and considerable differences to human insulin were found in both non-malignant and malignant cells.

Of note are the considerable inconsistencies between the findings of various studies that sometimes seemed contradictory. These discrepancies were most likely due to methodological differences between the various study settings and designs, such as the following:

The type of cells studied
It was shown that a high ratio of IGF-1 receptors to insulin receptors is required for a strong mitogenic effect of glargine [66]. It is likely that this applies not only to cell-lines, but also to primary cells, and to primary tumour cells and tissues. Considerable differences in receptor expression may exist between health and disease [67,68], and between individuals [16]. Many human cancers overexpress IGF-1 receptors, particular insulin receptors, and hybrid receptors 67, 69], in contrast to healthy tissues. Thus, cancer cell-lines or cancer tissue may be more susceptible to growth stimulation by insulin or insulin analogues than healthy tissue, and some cancer cell-lines may be more susceptible than others. Furthermore, metabolic potency studies may yield different results when performed in rat epididymal fat cells or in human subcutaneous fat cells [64].

The type of culture medium used

Some authors used high glucose concentrations, and some used low glucose concentrations in the cell cultures. The concentration of glucose may have a significant effect on the growth of cells in culture [70]. Moreover, the presence or absence of albumin, or of fetal calf serum (FCS) affects cell growth and the susceptibility of cells to growth factors or insulin: 10% FCS stimulates cell growth as much as does 10 nmol/l IGF-1 [5,63]. The content of insulin, oestrogen and IGF-1, for example, in FCS is rarely indicated by the manufacturer, and may vary between batches. Thus, starved cell cultures may yield different results compared to FCS-fed (i.e. maximally growing) cells in terms of stimulation by insulin or IGF-1.

Presence of albumin in the assay

The study data with detemir were particularly confusing because this analogue was sometimes compared to human insulin on an equimolar basis (which is inappropriate, because detemir in equipotent doses requires 4 times the molar concentration compared to human insulin). Moreover, in the presence of albumin the activity of detemir is dramatically reduced [54] and not all studies on detemir appropriately accounted for this phenomenon. Furthermore, the albumin species plays a role, for detemir binding to albumin differs according to the albumin species (i.e. it differs in dogs versus rabbits or pigs etc.). Hence, it could be argued that most of the previous *in vitro* studies comparing insulin detemir with other insulins are basically uninterpretable.

The concentration and the EC50 value of the ligand under study

The concentrations of insulin and the insulin analogues played a major role in the outcome of the experiments - low concentrations and high concentrations may yield qualitatively different cell responses. A comparison of bioactivities based on EC50 values rather than on equimolar concentrations might be preferable.

The duration of cell culture studies

The studies were carried out over varying time intervals, ranging from less than 1 hour to several days. In some studies, insulin or the insulin analogues were supplemented only once, whereas in other studies they were supplemented repeatedly (taking into account the fact that insulin degradation may occur in the culture). These aspects may have affected the performance of the assays.

The heterogeneity in methods and outcomes suggests that a standardization of the procedures for *in vitro* examinations of insulin analogues would be required in order to make the data more comparable. Whether these (and possible additional) *in vitro* differences in bioactivity between human insulin and the insulin analogues aspart, detemir, glargine, glulisine, and lispro translate into clinical outcomes remains to be elucidated. Patient populations with particular genetic make-up may be particularly susceptible [71]. There are already some indications that the *in vitro* differences might matter in clinical practice [33], and that other differences as disclosed by *in vivo* studies (e,g, glargine may increase serum IGF-1 concentrations in diabetic patients [72], detemir largely increases serum insulin levels [48,73] and may accumulate in the liver and the brain [74]) might matter, too.

In conclusion, the schematic presentation of the currently available *in vitro* data (except for receptor studies) on the differences in bioactivity between human insulin and the insulin analogues aspart, detemir, glargine, glulisine, and lispro displays a variety of abnormal activities of the analogues. The data- albeit suffering from heterogeneity between the assays-suggests that manipulation of the insulin molecule in order to improve its absorption from the subcutaneous tissue may cause unphysiological bioactivities with hitherto unknown consequences for the diabetic user as well as for the laboratory researcher.

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Authors' contributions
EAC did the search, EAC and HW participated in the screening and analysis of the data and in the writing of the final manuscript. All authors have read and approved the final version of the article.

Competing interests
The authors declare that they have no competing interests.

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